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# Intratumoral Predictive Markers in Metastatic Renal Cancer Patients

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### **Abstract**

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There is no established predictive marker for the treatment of metastatic renal cell cancer (mRCC) patients. With a predictive marker, patients unlikely to respond could be selected upfront and offered other therapy options. Thereby, unnecessary toxicity could be avoided and costs would be reduced. Tyrosine kinase inhibitors (TKI) are the cornerstone in the treatment of mRCC. The aim of the thesis was to evaluate and hopefully define tumoral predictive markers for treatment with the common TKIs sunitinib and sorafenib.

The studies are based on immunohistochemical analyses of renal cancer tissues from 139 primary tumors sampled in a tissue microarray. Three proteins with a specific and differential expression in RCC were chosen in co-operation with the Human Protein Atlas project. Since TKIs block vascular endothelial growth factor receptors (VEGFR) on tumor vessels, angiogenesis associated proteins were also analysed as putative predictive biomarkers.

In two studies, the renal proteins cubilin (CUBN) and pyruvate kinase L/R (PKLR) were investigated. Our results indicate that these membranous proteins are positive predictive factors for sunitinib and sorafenib therapies. Patients with high membranous expressions of CUBN and PKLR respectively experienced significantly longer progression free survivals (PFS) and overall survivals (OS) compared to the other patients. Combining CUBN and PKLR negative tumors, a patient group with a particularly short PFS could be defined, possibly consisting of patients not benefitting at all from treatment with sunitinib or sorafenib.

Studies of tumoral annexin A1 (ANXA1) and epidermal growth factor, latrophilin and seven transmembrane domain-containing protein 1 (ELTD1) demonstrated predictive potential for sunitinib but not for sorafenib treatment. A low cytoplasmic expression of ANXA1 was significantly associated with longer PFS and OS in patients treated with sunitinib. A combined analysis with CUBN and ANXA1 expression indicated a higher predictive value than the expressions of either marker alone. We further observed that a high vascular endothelial expression of ELTD1 is predictive for a longer PFS and OS in sunitinib treated patients. The expressions of CD34 which is a marker of the number of vessels and the sunitinib target VEGFR2 failed to demonstrate significant associations with PFS.

To conclude, our real world studies indicate that CUBN, PKLR, ANXA1 and ELTD1 are potential tumoral biomarkers, to predict benefit from treatment with sunitinib (all four proteins) and sorafenib (CUBN and PKLR) in patients suffering from mRCC.

*Keywords:* Renal cell cancer, predictive marker, tyrosine kinase inhibitor, tissue microarray, cubilin, annexin A1, PKLR, ELTD1

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*“She was afraid of heights  
but she was  
much more afraid  
of never flying.”*

*Atticus*

*Äidille ja iskille*



# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. **Niinivirta M.**, Enblad G., Edqvist P-H., Pontén F., Dragomir A., Ullenhag G. (2017)  
Tumoral cubilin is a predictive marker for treatment of renal cancer patients with sunitinib and sorafenib.  
*J Cancer Res Clin Oncol*, 143(6): 961-970
- II. **Niinivirta M.**, Enblad G., Edqvist P-H., Pontén F., Dragomir A., Ullenhag G. (2017)  
Tumoral ANXA1 is a predictive marker for sunitinib treatment of renal cancer patients.  
*J Cancer*, 8(19): 3975-3983
- III. **Niinivirta M.**, Enblad G., Lindskog C., Pontén F., Dragomir A., Ullenhag G.  
Tumoral pyruvate kinase L/R as a predictive marker for the treatment of renal cancer patients with sunitinib and sorafenib.  
Submitted
- IV. **Niinivirta M.**, Georganaki M., Enblad G., Lindskog C., Dimberg A., Ullenhag G.  
Tumor endothelial ELTD1 as a predictive marker for treatment of renal cancer patients with sunitinib.  
Submitted

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# Contents

Introduction.....	11
Epidemiology .....	11
Aetiology.....	11
Symptoms.....	12
Diagnosis.....	12
Pathology.....	12
Classification.....	13
Prognostic factors.....	13
Treatment.....	15
Localised RCC: surgery and adjuvant therapy.....	15
Metastatic RCC: surgery and metastasectomy.....	16
Metastatic RCC: systemic treatment.....	16
Targeted therapies.....	17
Immunotherapy.....	19
Metastatic RCC: radiotherapy.....	20
Metastatic RCC: other pharmacologic agents.....	20
Predictive markers.....	22
Serum markers.....	22
Tissue markers.....	23
Clinical markers.....	24
Studied proteins.....	26
Cubilin (CUBN).....	26
Annexin A1 (ANXA1).....	26
Pyruvate kinase liver and red blood cells (PKLR).....	27
Epidermal growth factor, latrophilin and seven transmembrane domain-containing protein 1 (ELTD1).....	28
Aims of the thesis.....	30
Overall aim.....	30
Specific aims.....	30

Materials .....	31
Paper I, II, III and IV .....	31
Paper I and III .....	32
Paper II and IV .....	33
Methods .....	34
Paper I, II, III and IV .....	34
TMA generation .....	34
Immunohistochemistry Paper I, II and III .....	34
Immunohistochemistry and slide scanning in Paper IV .....	34
Slide scanning and evaluation of staining in Paper I, II and III .....	35
Image analysis in Paper IV .....	35
Results .....	37
Paper I .....	37
Paper II .....	37
Paper III .....	38
Paper IV .....	38
Discussion .....	40
Conclusions .....	44
Future perspectives .....	45
References .....	49

# Abbreviations

ANXA1	Annexin A1
CAIX	Carbonic anhydrase IX
cfDNA	Circulating cell-free DNA
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CUBN	Cubilin
EC	Endothelial cell
EGFR	Epidermal growth factor receptor
ELTD1	EGF, latrophilin and seven transmembrane domain-containing protein 1
FLT-3	Fms like tyrosine kinase 3
HFSR	Hand-foot skin reaction
HIF	Hypoxia-inducible factor
IFN- $\alpha$	Interferon alfa
IL-2	Interleukin 2
IMDC	International Metastatic RCC Database Consortium
MMP	Metalloproteinase
MSKCC	Memorial Sloan-Kettering Cancer Center
mRCC	Metastatic RCC
mTOR	Mammalian target of rapamycin
MVD	Microvascular density
NGAL	Neutrophilgelatinase-associated lipocalin
NSS	Nephron-sparing surgery
OS	Overall survival
PDGF	Platelet-derived growth factor
PD-L1	Programmed death-1 ligand
PFS	Progression free survival
PKLR	Pyruvate kinase liver and red blood cells
RANKL	Receptor activator of nuclear factor $\kappa$ -B ligand
RCC	Renal cell carcinoma
SciLifeLab	Swedish Science for Life Laboratory
TGF- $\beta$	Transforming growth factor $\beta$
TKI	Tyrosine kinase inhibitor
TMA	Tissue microarray
TNF $\alpha$	Tumor necrosis factor $\alpha$
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau



# Introduction

## Epidemiology

Renal cell carcinoma (RCC) accounts for 2-3% of all adult malignancies, representing the seventh most common cancer in men and tenth most common cancer in women <sup>1</sup>. Worldwide, there are about 338.000 new cases and 102.000 deaths per year <sup>2</sup>. Around 1000 cases of RCC are diagnosed in Sweden each year with 1.5 to 2 times more male compared to female patients. The majority of the patients are over 65 years (The Swedish National Board of Health and Welfare).

Earlier one third of the patients had metastatic disease at the time of the diagnosis <sup>3</sup>. Nowadays several of the RCCs are diagnosed incidentally due to frequent use of imaging methods and only about 20% have a metastatic disease at diagnosis. Moreover, 20% of primary cured RCC patients relapse within 5 years. Spread can be both lymphatic and haematogenous. RCC metastases are most often localized to the lungs, lymph nodes, bone, liver and brain <sup>1 3</sup>.

The overall survival (OS) has increased in Sweden during the last decade. Overall survival associates with the stadium of RCC at diagnosis. For localized disease with small tumors, the relative 5-years-survival is over 90% compared to 70% for the whole group. The 5-years OS for primary metastasized patients is around 15% in Sweden (The Swedish National Board of Health and Welfare 2013 and 2017). Worldwide, the prognosis is still poor for metastatic RCC (mRCC) patients, the estimated average 5-years-survival rate is 8% <sup>4</sup>.

## Aetiology

Identified aetiological factors are mainly related to lifestyle, such as smoking, obesity and hypertension. Incidence increases consistently with age <sup>5-7</sup>. Approximately 2-3% of RCC are hereditary and several autosomal dominant syndromes are described, the most common one being Von Hippel Lindau disease (VHL) <sup>2</sup>. The disease is caused by mutations in VHL-tumor suppressor gene on chromosome 3. Patients with VHL can develop hemangioblastomas on the brain and retina, renal cysts and carcinomas, pancreatic cysts and neuroendocrine tumors <sup>8</sup>.

## Symptoms

Many renal cell tumors are asymptomatic until the later stages of the disease. The three classical symptoms, haematuria, flank pain and a mass in the abdomen or flank, are nowadays rare and more than 50% of RCCs are discovered as mentioned earlier accidentally with ultrasound (US), computerised tomography (CT) and magnetic resonance imaging (MRI)<sup>9</sup>.

Paraneoplastic syndromes are detected both in localized and mRCC. Symptoms and signs can include hypertension, pyrexia, night sweats, anaemia, hypercalcaemia, elevated erythrocyte sedimentation rate and weight loss<sup>3</sup>.

## Diagnosis

Computer tomography of the abdomen, pelvis and chest with and without contrast is used to characterize a renal mass and to assess whether metastases are present. In case of affected kidney function or allergy towards contrast medium a MRI or UL are valid options.

If the patient has any abnormal clinical or laboratory signs or symptoms other diagnostic procedures may be used, such as brain CT or bone scan, to identify possible metastases<sup>10</sup>.

A renal tumor core biopsy provides histopathological confirmation of malignancy. Nephrectomy is sometimes indicated without prior biopsy. For patients with metastatic disease biopsy is indicated from the primary tumor, alternatively from a metastatic lesion to ensure the diagnosis, if systemic therapy is considered without prior nephrectomy.

## Pathology

Renal cell cancer is the most common solid lesion within the kidney and accounts for approximately 90% of renal malignancies<sup>9</sup>. Clear-cell RCC is the most frequent subtype of sporadic RCC in adults (80-90%). Two other major histologic RCC types are papillary RCC (10-15%) and chromophobe RCC (4-5%)<sup>9</sup>. Minor histologic RCC types are for example collecting duct RCC (Bellini tumours) and sarcomatoid RCC<sup>2</sup>.

The most popular and widely used pathological grading system for RCC is the "Fuhrman Nuclear Grade". It is a system based on assessment of the uniformity of nuclear size, nuclear shape and nuclear prominence. Nuclear characteristics used in the Fuhrman Grade particularly indicate how active the cells are in making protein. Fuhrman scale is graded I-IV, where grade I does not differ much from ordinary renal cells, with small and round nuclei

without nucleoli and carries the best prognosis. With growing grade the tumor cells become more different from normal renal cells. Grade IV consists of tumor cells with bizarre nuclei and clumps of chromatin. This grade is related to the worst prognosis <sup>11</sup>.

In at least 60% of sporadic clear cell RCC, VHL gene is mutated and inactivated. VHL is a tumor suppressor gene and encodes the VHL-protein <sup>12</sup>. Von Hippel-Lindau protein promotes degradation of hypoxia-inducible factor  $\alpha$  (HIF- $\alpha$ ). Abnormal VHL gene results in default VHL-protein and the levels of HIF- $\alpha$  increase which results in overexpression of vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). Vascular endothelial growth factor and PDGF bind to and activate their receptors on the surface of vessels resulting, especially in the case of VEGF, in elevated vascular permeability and endothelial cell migration which improve tumor angiogenesis, tumor growth and metastasis <sup>12 13-15</sup>.

## Classification

The current TNM classification system is recommended for clinical and scientific use. T1 ( $\leq 7$  cm) and T2 ( $> 7$  cm) tumors are limited to the kidney whereas T3-4 tumors are locally advanced, involving structures like major veins, perirenal fat and ipsilateral adrenal gland. Based on the TNM classification, the disease is grouped into stages (Table 1) <sup>1</sup>.

Table 1. *Staging of RCC* <sup>1</sup>

Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1-2	N1	M0
	T3	Any	M0
Stage IV	T4	Any	M0
	Any	Any	M1

## Prognostic factors

The aim of prognostic factors in general is to predict the outcome of patients, risk of recurrence and death, independent from the treatment choice.

In localized RCC many different factors are used to assess prognosis, among them tumor size, tumor histology, Fuhrman's grade, tumor necrosis, Karnofsky performance status and TNM <sup>1</sup>.

In the metastatic setting, five prognostic factors for predicting survival were identified after studying 670 patients with advanced RCC in 24 Memorial Sloan-Kettering Cancer Center (MSKCC) clinical trials between 1975

and 1996. Risk factors associated with a shorter survival period were low Karnofsky performance status, high lactate dehydrogenase, low serum hemoglobin, high serum calcium and absence of prior nephrectomy. According to these risk factors, patients were stratified into three different groups; favourable-risk (no risk factors), intermediate-risk (one or two risk factors) and poor-risk (three or more risk factors). Three-year survival percentages were 31%, 7% and 0%, respectively <sup>16</sup>. The updated version in 2002 was based on a study of nearly 500 mRCC patients treated with interferon- $\alpha$  (IFN- $\alpha$ ). Prior nephrectomy was replaced by the time from the primary diagnosis of RCC to treatment, considered as a risk factor if less than one year <sup>17</sup>. Memorial Sloan-Kettering Cancer Center criteria were established before the era of immunotherapy and new therapies. Because of advances in the systemic cancer treatment of mRCC, existing MSKCC criteria were revalidated in 2009. Over 600 patients with mRCC treated with sunitinib, sorafenib or bevacizumab plus interferon from August 2004 to July 2008 were included in this study. Four prognostic factors (Karnofsky performance status, time from primary diagnosis to treatment, hemoglobin and serum calcium) were equal from MSKCC criteria. The new Hengs criteria (also called as International Metastatic RCC Database Consortium IMDC criteria) also include neutrophil count and platelet count. Two-year survival percentages were 75% in the favorable-risk group, 53% in the intermediate-risk group and 7% in the poor-risk group <sup>18</sup>.

# Treatment

## Localised RCC: surgery and adjuvant therapy

Surgery is the only curative therapeutic approach for RCC. Nephron-sparing surgery (NSS) (partial nephrectomy) for localised RCC is generally recommended for T1-tumors<sup>19</sup>. Also patients with one kidney, renal insufficiency, hereditary RCC or bilateral tumors should be candidates for NSS<sup>20</sup>. In patients with locally advanced tumors, radical nephrectomy is indicated, either by laparoscopic or open surgery<sup>21</sup>.

Lymph node dissection does not prolong long-term survival following nephrectomy but is needed for staging purposes<sup>22</sup>.

There is no role for systemic adjuvant therapy after surgery outside controlled clinical trials<sup>23</sup>. An autologous renal tumor cell vaccine have shown a significant positive impact on PFS compared to a control group with no adjuvant treatment after radical nephrectomy in a phase III study<sup>24</sup>. This treatment is still not clinically established. A study of almost 2000 patients with intermediate-, high-, and very high-risk completely resected RCC receiving sunitinib, sorafenib (both described in detail under “Targeted therapies”) or placebo for up to one year did not show improved OS<sup>25</sup>. Another large study with over 1500 patients receiving pazopanib or placebo after surgery showed no significant difference in disease free survival between study groups<sup>26</sup>. Though, in the S-TRAC trial adjuvant sunitinib (n=309) showed benefit over placebo (n=306) in patients with high risk of recurrent RCC, sunitinib treated patients experiencing 1.2-year longer time before the disease recurred<sup>27</sup>. After these conflicting results, the gain of one year sunitinib-treatment is not consider to outweigh the harm and the costs it means and thereby adjuvant therapy is still not recommended<sup>28</sup>. Pembrolizumab, a programmed death 1 (PD-1) inhibitor (see more in detail under “Immunotherapy”), is under investigation in the adjuvant setting for RCC patients after nephrectomy. Overall, many studies are ongoing with different kinds of therapies and adjuvant treatment might have a role in the future<sup>1</sup>.

Patients with localized RCC are controlled regularly after nephrectomy both clinically and with CT of chest and abdomen as well as with blood samples such as hemoglobin, sedimentation rate and renal function. Magnetic resonance imaging or ultrasound is a better choice to CT in younger patients to

avoid radiation exposure, in patients with abnormal kidney function and in patients with allergy to CT contrast medium.

## Metastatic RCC: surgery and metastasectomy

Eighty-five percent of metastases are diagnosed within 3 years and about 93 % within 5 years after nephrectomy<sup>29</sup>. As earlier mentioned, the most common localizations for metastatic spread are lungs, lymph nodes, bone, liver, brain and adrenal gland<sup>20</sup>.

In routine practice, radical nephrectomy (also referred to as debulking or cytoreductive nephrectomy) has been recommended for patients in good performance status with adequate cardiac and pulmonary function and have most of the tumor burden (>75%) in the involved kidney<sup>30</sup>. In rare patients removal of the primary renal tumor leads to spontaneous regression of the metastatic disease<sup>31</sup>. As the standard treatment of mRCC has changed towards targeted therapies the role of cytoreductive surgery has been questioned. In a recently published study sunitinib alone was non-inferior to surgery followed by sunitinib in intermediate or poor risk mRCC patients<sup>32</sup>. Though, mRCC patients earlier suggested as candidates to nephrectomy (intermediate-risk and low tumor burden), were under represented in this study. Which primary treatment is to prefer requires further studies but highlights the importance of patient selection in each case.

Selected patients with mRCC can be considered for metastasectomy. Favourable prognostic factors include solitary or easily accessible pulmonary metastases, solitary extricable intra-abdominal metastases, a long disease-free interval after nephrectomy, or a good response to targeted therapy<sup>33,34</sup>.

## Metastatic RCC: systemic treatment

Treatment of mRCC patients with chemotherapy has been unsuccessful and sensitivity to radiotherapy is very limited<sup>3</sup>. Metastatic renal cell cancer was earlier, still only 10 years ago, treated with cytokine therapy (IFN- $\alpha$  and high-dose interleukin 2 [IL-2]), IFN- $\alpha$  being the most frequently used cytokine. Objective response rate for IFN- $\alpha$  is only 7.5-12% and OS in median 13 months<sup>3,35</sup>. Combined therapy with both IFN- $\alpha$  and IL-2 resulted to higher response rate (18.6%) but also caused a significant toxicity which limited the use of both cytokines together<sup>35</sup>. Hence, with the traditional immunotherapy, complete and partial tumor regressions were achieved in a minority of patients and RCC became early considered as an immunogenic malignancy<sup>35,36</sup>.

## Targeted therapies

Sunitinib is an oral tyrosine kinase inhibitor (TKI) which selectively inhibits the PDGF receptors, the VEGF receptors 1-3, c-KIT and fms like tyrosine kinase 3 (FLT-3)<sup>14</sup>. In the first line setting in selected mRCC patients, the median progression free survival (PFS) extended to 11 months for sunitinib while patients treated with IFN- $\alpha$  experienced a PFS of 5 months ( $p < 0.001$ ). Patients treated with sunitinib had a median OS of 26.4 months versus 21.8 months for interferon-treated patients ( $p = 0.051$ )<sup>37</sup>. Sunitinib is recommended as standard of care in the first line setting for mRCC<sup>20</sup>.

Sorafenib is another oral TKI but opposed to sunitinib it also inhibits an intracellular signaling enzyme, Raf-kinase<sup>38</sup>. In the second line setting in patients with mRCC, the median PFS extended to 5.5 months for sorafenib while patients treated with placebo in the same trial experienced a PFS of 2.8 months ( $p < 0.01$ )<sup>39</sup>. Sorafenib is an option of treatment for selected patients in front line and as subsequent therapy for patients with predominantly clear cell mRCC<sup>20</sup>.

Pazopanib is an oral angiogenesis inhibitor targeting VEGFR, PDGFR and c-KIT. In treatment naive and cytokine treated mRCC patients pazopanib was compared to placebo. Five months longer PFS was observed for pazopanib treated patients (9.2 months versus 4.2 months)<sup>40</sup>. Comparing pazopanib and sunitinib in a study of over 1000 patients resulted in no difference in PFS or OS but pazopanib treated patients experienced better health-related quality of life. The side-effects varied between these two agents, sunitinib associated more with skin toxicity and pazopanib with liver toxicity.<sup>41</sup> Pazopanib is one of the preferred first-line medications for mRCC<sup>1,20</sup>

Cabozantinib, one of the newest oral TKIs in mRCC treatment arsenal, inhibits VEGFR2, c-MET and AXL. The results from a study comparing capozantinib with everolimus in mRCC patients who have experienced disease progression on previous VEGFR TKI-treatment resulted in recommendation to use capozantinib in the second line setting. Progression free survival and OS were significantly prolonged and the response rate significantly improved<sup>42</sup>. In a latter study, capozantinib was found to prolong PFS and OS as first line treatment in intermediate- and high-risk mRCC patients compared with sunitinib therapy (PFS 8.2 months versus 5.6 months, OS 30.3 months versus 21.8 months). Complete or partial responses were higher with capozantinib (33% versus 12%). Patients experienced the same level of side effects in both treatment arms (grade 3-4 adverse events 67% versus 68%). Though, quality of life data was not collected in this study<sup>43</sup>.

The arsenal of TKIs is growing fast and other used therapies include e.g. axitinib and lenvatinib.

There are several side-effects of TKI-treatment and one of the most common is hypertension. The proposed mechanism behind this is inhibited syn-

thesis of nitric oxide, a known vasodilator, and stimulated endothelin-1 pathway, promoting vasoconstriction<sup>44</sup>. Hand-foot skin reaction (HFSR) is another common seen side effect, which exact pathogenesis is unknown<sup>45</sup>. Patients treated with TKIs can also suffer from fatigue, bone marrow toxicity, mucosal toxicity and diarrhea. Sunitinib can also cause hypothyroidism<sup>46</sup>.

Mammalian target of rapamycin (mTOR) inhibitors like everolimus (oral mTORi) and temsirolimus (i.v.) inhibit angiogenesis and proliferation<sup>47</sup>. In a trial with everolimus versus placebo in mRCC patients, who had failed targeting therapy, the PFS was 4 months with everolimus versus 1.9 months with placebo ( $p < 0.0001$ )<sup>48</sup>. In another phase III study, poor-risk mRCC patients were randomized to receive first-line treatment with temsirolimus or standard treatment with IFN- $\alpha$  monotherapy or the combination. The median OS was significantly longer in the monotherapy temsirolimus group, 10.9 versus 7.3 months in the IFN- $\alpha$  group ( $p < 0.0069$ ). There was no survival benefit for the patients in the combination treatment arm compared to standard treated patients<sup>49</sup>. In clinical routine today, everolimus in combination with a wide range targeting TKI, lenvatinib, is approved for mRCC-patients who progressed after one previous VEGF-treatment<sup>50</sup>.

Mammalian target of rapamycin inhibitors are associated with immunosuppression, metabolic alterations (hyperglycemia, hypertriglyceridemia, hypercholesterolemia) and interstitial pneumonitis<sup>46</sup>.

Vascular endothelial growth factor is a protein family of growth factors consisting of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta-derived growth factor. These proteins play an important role in both physiological and pathological angiogenesis. By binding to and activating, through phosphorylation, their tyrosine kinase receptors (VEGFR1-3) on the endothelial cell surface they induce many cellular processes leading to new vessels<sup>51</sup>. Hypoxia-inducible factor  $\alpha$ , which is tightly regulated by oxygen availability, stimulates, as previously mentioned, especially release of VEGF-A<sup>13</sup>. Bevacizumab is a monoclonal antibody that binds and neutralizes circulating VEGF-A protein<sup>52</sup>. Inhibition of VEGF-A leads to inhibition of angiogenesis. In first line setting, mRCC patients were treated with INF- $\alpha_2 \pm$  bevacizumab in a phase III trial. PFS was 10.2 months in the combination arm versus 5.4 months for the group not receiving bevacizumab ( $p < 0.0001$ )<sup>53</sup>. Common side-effects of bevacizumab are hypertension, proteinuria and bleeding. Gastrointestinal perforation is a rare but life-threatening event associated with bevacizumab.

## Immunotherapy

Programmed death 1 is a checkpoint protein which receptor is expressed on the surface of T-cells. Its ligand, programmed death-ligand 1 (PD-L1), is expressed on dendritic cells and macrophages. When PD-L1 binds to PD-1, T-cells are blocked and the whole immune response is inhibited. Programmed death-ligand 1 is overexpressed on tumor cells and in this way cancer cells have the ability to resist the immune system. To overcome this, key checkpoint inhibitors were developed. The human PD-1 antibody nivolumab significantly prolonged OS compared with everolimus (25.0 versus 19.6 months) as second line treatment after TKI therapy in a study of over 800 patients. The objective response rate was also significantly different in favour of nivolumab (25% versus 5%). Though, no significant difference in PFS could be found (4.6 versus 4.4 months). Nivolumab treatment is nowadays considered as a standard in the 2<sup>nd</sup> line setting after prior VEGF targeted therapy<sup>54</sup>.

As the next step, the combination of PD-1 inhibitor (nivolumab) and cytotoxic T-lymphocyte associated protein 4 (CTLA4) (ipilimumab) were compared to single sunitinib treatment in previously untreated clear cell mRCC patients (n= > 1000). Already 2007 Yang published a study of ipilimumab effect in mRCC patients, though with considerable toxicity which precluded its further development as monotherapy<sup>55</sup>. In this new combination study, ipilimumab was given only as four doses together with nivolumab, thereafter continuing with nivolumab single therapy. Significantly higher objective response rates were measured (42% versus 27%) as well as longer PFS (11.6 months versus 8.4 months) in the combined immunotherapy arm compared to sunitinib arm. The subgroup analysis revealed that the combination therapy was better in intermediate- and poor risk subset. Meanwhile, the opposite result was observed in the favorable risk subset with higher response rate and longer PFS in sunitinib-treated group (52% and 25.1 months versus 29% and 15.3 months). Moreover, patient subset with expression of PD-L1  $\geq$  1% of tumor cells showed considerably higher response rate and PFS when treated with immunotherapy compared to sunitinib-treatment (58% and 22.8 months versus 25% and 5.9 months). In the sunitinib-treated group, patients with < 1 % PD-L1 expression experienced prolonged PFS compared to patients with positive expression of PD-L1 (10.4 versus 5.9 months, respectively). Forty six percent of patients treated in the combination arm developed grade 3 and 4 adverse events. In the sunitinib group the same occurred in 63%. Though, the treatment was discontinued in 22% of nivolumab-ipilimumab arm but only in 12% of patients in the sunitinib arm due to treatment related adverse events<sup>56</sup>. The combined immunotherapy is in clinical use in USA for intermediate and poor-risk mRCC patients as a front-line therapy but not in Sweden.

Combining multitargeted TKIs and PD-1 checkpoint inhibitors has resulted in clinical benefit but also in unacceptable toxicity. A more selective TKI, axitinib, targeting VEGFR 1-3, was combined first with pembrolizumab showing antitumoral activity with tolerable side effects <sup>57</sup>. In an ongoing study, treatment naïve mRCC patients receive either the combination of axitinib plus pembrolizumab or single sunitinib. In general, a large number of trials are currently investigating the combination of “newer” TKIs and immunotherapy.

## Metastatic RCC: radiotherapy

Bone metastases are rather common, 30-40% of mRCC patients suffer from them <sup>20</sup>. Radiotherapy is used to reduce symptoms such as pain, to stabilize skeleton in case of threatening spontaneous fracture and towards spinal cord compression, if surgery is not possible. Two-thirds of patients with symptomatic bone metastases experience pain relief after radiotherapy <sup>2</sup>.

When a patient suffers from a single brain lesion, a neurosurgical operation should be discussed. Local recurrence is observed more rarely when the surgical resection is followed by radiation compared to radiotherapy alone <sup>58</sup>. Another alternative for patients with a single brain metastasis is stereotactic cranial radiotherapy where high doses of radiation reach the target and surrounding healthy tissue only gets low doses. Gamma Knife can be used in this situation <sup>59</sup>. Patient with multiple brain metastases is usually offered whole brain irradiation, at a total dose of 20 to 30 Grey in 4-10 fractions <sup>1</sup>.

## Metastatic RCC: other pharmacologic agents

Biphosphonate therapy with zoledronic acid reduces skeletal-related events in patients with bone metastasis due to mRCC <sup>60</sup>. Renal cancer associated hypercalcaemia is treated with i.v. bisphosphonate. Zoledronic acid should be considered in these cases but treatment requires adequate renal function and administration intravenous access.

Denosumab, a human monoclonal antibody, can also be used to treat hypercalcemia. It binds and inhibits receptor activator of nuclear factor  $\kappa$ -B ligand (RANKL) with high affinity. Receptor activator of nuclear factor  $\kappa$ -B ligand is released in the bone when metastatic tumor cells are present. It activates osteoclasts leading to increased bone resorption. Denosumab is given as subcutaneous injections and it is not cleared by the kidneys and therefore requires no dose modification in renal impairment <sup>61</sup>.

Corticosteroids are in line with other solid malignancies used as a palliative therapy to stimulate appetite, to prevent weight loss and for general well-being. They are also used for patients with spinal cord compression to

reduce oedema and for brain metastases to relieve cerebral symptoms at least temporary <sup>1</sup>.

# Predictive markers

Predictive markers intend to assess the likelihood of a patient responding before receiving a specific therapy. They thus indicate the sensitivity or resistance to a specific therapy already upfront.

There are predictors in clinical use for some other malignancies. In breast cancer expression of estrogen and progesterone receptors identifies patients with expected benefit of hormone therapy and human epidermal growth factor receptor 2 (HER2) guides the treatment choice of trastuzumab<sup>62</sup>. In metastatic colorectal cancer ras-oncogene analysis defines patients with mutation as resistant to epidermal growth factor receptor (EGFR) inhibitors (cetuximab, panitumumab)<sup>63</sup>. The opposite is seen in patients with advanced or metastatic non-small cell lung carcinomas (NSCLC): EGFR mutant tumors predict treatment response for gefitinib and erlotinib, TKIs targeting EGFR, and are recommended instead of chemotherapy, as first line therapy for this patient group<sup>64</sup>. Patients with wild type EGFR are further analyzed for anaplastic lymphoma kinase (ALK) rearrangements. Crizotinib, an ALK-inhibitor, is used for NSCLC-patients with ALK-positive tumors resulting in impressive PFS and objective response rate<sup>65</sup>. Moreover, patients with BRAF mutated metastatic melanoma gain from vemurafenib and dabrafenib which are two widely used BRAF-inhibitors<sup>66</sup>. Later on these targeting agents have been combined with MEK-inhibitor trametinib to undergo resistance to monotherapy with BRAF-inhibitors<sup>67</sup>.

In mRCC there are no known predictive markers for TKI treatment in clinical use. Targeting therapies can cause many side-effects, sometimes chronic toxicity and adverse events. Considering this and the economic burden of TKIs, predictive biomarkers are really needed. Moreover, some patients do not benefit from the treatment at all and with a predictor this group of patients could be identified already before treatment start.

## Serum markers

Serum is the easiest way to analyse expression of proteins that can be used as biomarkers. In our case we have focused on markers with potential predictive value. Blood tests are easy to take and easy to follow. While heterogeneity may be confounding in tumor based analysis, serum proteins could reflect the average of the tumor load. The majority of the studies published

have investigated potential predictors for response only to sunitinib therapy. Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) is supposed to act as a growth factor and metalloproteinase-9 (MMP-9) mobilizes VEGF thereby both supporting cancer development<sup>68,69</sup>. In a subset of 21 sunitinib-treated patients the group of non-responders showed significantly elevated TNF- $\alpha$  and MMP-9 levels at the baseline. Moreover, reduced time to progression (TTP) and OS were also significantly correlated with erased pre-treatment levels of these two proteins<sup>70</sup>.

Baseline levels of VEGF and neutrophilgelatinase-associated lipocalin (NGAL) in serum have also been proposed as predictors for sunitinib-therapy in mRCC. Neutrophilgelatinase-associated lipocalin is observed in cells during different kinds of stress, such as inflammation and cancer. Patients with high VEGF and NGAL pre-treatment levels experienced a higher relative risk of disease progression and significantly shorter PFS in a study of 85 sunitinib treated mRCC patients<sup>71</sup>.

Published studies investigating possible predictive factors for sorafenib treatment are few. The effect and safety of sorafenib treatment towards placebo was studied in a cohort of over 900 patients. In a subanalysis, the association between levels of VEGF and PFS was assessed. The study indicated that patients with elevated VEGF-levels at treatment start experienced greater benefit from sorafenib treatment<sup>72</sup>. Circulating cell-free DNA (cfDNA) is supposed to originate from tumors due to apoptosis and necrosis and higher levels are detected in cancer patients compared to healthy controls. In a study of 18 mRCC patients, no correlation between baseline cfDNA and response to sorafenib treatment was found. However, in patients with remission or stable disease, significantly lower levels of cfDNA were noticed during medication (week 8-24) compared to those with progressive disease<sup>73</sup>.

## Tissue markers

Studying the tumor tissue is the most accurate way to investigate protein expressions. Tissue microarray (TMA) is a paraffin block in which more than hundred tissue cores are assembled and can be analyzed rapidly<sup>74</sup>. One major advantage of TMA-based studies is that all the cores are stained at the same time and under same conditions ruling out intertest variabilities.

In a TMA from primary tumor tissue of 42 mRCC patients several angiogenesis-associated proteins were studied as potential predictive biomarkers in relation to response to sunitinib. A high HIF-1 $\alpha$  score and VEGFR3 vessel staining showed significant association with longer PFS. Furthermore, VEGFR1 and -3 vessel staining, low PDGFR $\alpha$  score and high CA9 score were positively correlated to a longer OS. Median OS did differ significantly between patients with high and low CA9 expression (48 versus 22 months, respectively)<sup>75</sup>.

Carbonic anhydrase IX (CAIX) is overexpressed due to hypoxia in RCC and is most often absent in benign renal tissue <sup>76</sup>. It has been proposed as a predictor for both sunitinib and sorafenib response. Studying 94 clear cell mRCC-patients sunitinib treated (n=39) showed no tendency to correlation between CAIX expression and response. However, high CAIX expression was associated with greater response to sorafenib (n=40), measured as tumor shrinkage (p= 0.05) <sup>77</sup>. Conflicting results were published in a study of 133 sorafenib (n= 66) and placebo treated patients where no evidence of tumor CAIX expression as a predictor in this patient cohort was found <sup>78</sup>. In another study, more objective responses were observed in sunitinib and sorafenib (n=53) treated patients with elevated membranous CAIX expression (p= 0.004) <sup>79</sup>.

In a large TMA, constructed of tumor tissue from over 400 advanced RCC patients receiving either pazopanib or sunitinib, tumoral expression of PD-L1 was evaluated. Patients with increased tumor cell PD-L1 or PD-L1 plus tumor CD8-positive T-cell counts had significantly shorter PFS and OS <sup>80</sup>.

In 96 sorafenib treated mRCC patients the direct targets of sorafenib treatment as well as the tumor vasculature were investigated. Microvessel area (MVA) represented the vasculature and was measured by CD34 staining. Only a high MVA was associated with a higher likelihood of response to the treatment, the other studied expressions showed no predictive value <sup>81</sup>.

Table 2. *Published studies of potential predictors for treatment of mRCC*

Treatment	Serum	Tumor tissue
Sunitinib	TNF- $\alpha$ , MMP-9 (n=21) <sup>70</sup>	HIF-1 $\alpha$ , CD31, VEGFR1 VEGFR3, PDGFR $\alpha$ , CA9 (n=42) <sup>75</sup>
	VEGF, NGAL (n=85) <sup>71</sup>	PD-L1 (n=453) <sup>80</sup>
Sorafenib	VEGF (n= 708) <sup>72</sup>	CAIX (n=40) <sup>77</sup>
	cfDNA (n=18) <sup>73</sup>	MVA (n=96) <sup>81</sup>

## Clinical markers

As mentioned earlier, one of the most common side effects of sunitinib treatment is hypertension. Patients with mRCC developing hypertension during sunitinib therapy seem to gain more from the treatment with prolonged PFS and OS <sup>82</sup>. The risk of disease progression was almost five times

lower in patients with treatment related hypertension in a study of over hundred patients <sup>82</sup>. Another study suggested hypertension as an independent predictor of PFS <sup>83</sup>. In a cohort of over 500 sunitinib treated mRCC patients significantly improved PFS and OS were measured in patients with treatment related hypertension (PFS 12.5 versus 2.5 months, OS 30.9 versus 7.2 months) <sup>84</sup>. The anti-hypertensive medication is usually initiated during the first month of sunitinib treatment (median 28 days from start) <sup>83</sup>.

Development of hypertension also during sorafenib treatment in 148 patients was noticed as a marker for early response assessment <sup>85</sup>.

The majority of skin reactions caused by sunitinib and sorafenib are noted already during the first treatment cycle and can therefore be used as an early evaluator of treatment response <sup>86</sup>. A study of over 1000 patients with mRCC treated with sunitinib (n=705) and sorafenib (n=365) showed improved PFS and OS associated with HFSR due to sunitinib. Patients treated with sorafenib and suffering from HFSR could only reach a trend towards same correlation <sup>87</sup>. However, in a much smaller study of 36 patients treated with sorafenib, patients (n=23) experiencing HFSR had significantly longer PFS compared to the rest (n=13) <sup>88</sup>.

Though, as commented before, both hypertension and HFSR are clinical findings that occur only after treatment start and cannot be used as pre-treatment predictors. The majority of patients suffer from these side-effects early during the treatment course and therefore they can instead be used as early evaluators of response.

# Studied proteins

## Cubilin (CUBN)

Cubilin is an endocytic multi-ligand receptor located within the epithelium of ileum, proximal renal tubules and visceral yolk sac. Cubilin acts in the small intestine as a receptor for intrinsic factor-vitamin B12 complexes mediating the uptake of vitamin B12<sup>89</sup>. Together with megalin CUBN reabsorbs a wide variety of filtered proteins and vitamins like albumin, vitamin D-binding protein, transferrin, lipoprotein and other plasma carriers in the membrane of proximal renal tubule<sup>90</sup>.

Patients with hereditary Imerslund-Gräsbeck syndrome suffer from malabsorption of vitamin B12 leading to juvenile onset of megaloblastic anemia. Abnormalities in the CUBN gene are supposed to cause the syndrome<sup>91</sup>. Not only vitamin B12 malabsorption is seen in these patients but also proteinuria consisting of CUBN ligands mentioned above. The lack of B12-vitamin can be corrected by parenteral injections but the proteinuria not<sup>89</sup>.

Cubilin has shown highly tissue specific expression patterns in normal kidney and in RCC as assessed on both by immunohistochemistry and at the RNA level and was therefore selected as a potentially interesting protein to investigate in our study ([www.proteinatlas.org](http://www.proteinatlas.org)).<sup>92</sup>

Cubilin's function in cancer is unknown. Though, lack of CUBN expression has shown correlation with poor prognosis and therefore is supposed to have a prognostic value in RCC<sup>93</sup>.

## Annexin A1 (ANXA1)

Annexin A1 is part of a protein family of annexins, having in common a calcium binding site which enables them to bind (i.e. to annex) to negatively charged membrane phospholipids<sup>94</sup>. Annexin A1 was earlier called as lipocortin and was the first characterized member of the annexin family. Lipocortin was found to act as a direct phospholipase A2 (PLA2) inhibitor, released by stimulation of glucocorticoids used as medication towards inflammation. By suppressing PLA2, the production of prostaglandins and leukotrienes, potent mediators of inflammation, are reduced<sup>95</sup>. Annexin A1 is expressed at high levels in the cytoplasm of resting human immune cells like monocytes, neutrophils and macrophages. It is rapidly mobilized to the cell

surface and secreted when the immune cells are activated. The secreted extracellular ANXA1 is supposed to have many anti-inflammatory properties: firstly, ANXA1 improves neutrophil detachment to the site of inflammation. Second, by inhibiting neutrophil and monocyte extravasation through the endothelium of blood vessels the accumulation of leukocytes at the inflammatory site is limited. Thirdly, ANXA1 also, later on, induces the neutrophils to die by apoptosis and finally promotes the clearance of apoptotic cells by phagocytosis <sup>96</sup>.

Endothelial cell migration, which is stimulated by VEGF, is one of the required steps in the angiogenesis. Phosphorylation of ANXA1 is suggested to be induced by VEGF and promote cell migration and therefore ANXA1 might own pro-angiogenetic qualities <sup>97</sup>. Moreover, ANXA1 is proposed to stimulate transforming growth factor  $\beta$  (TGF- $\beta$ ), a known inducer of invasion, which further upregulates MMP2 and MMP9. MMPs degrade extracellular matrix allowing tumor spread <sup>98,99</sup>. That said, ANXA1 surely plays a role in tumor invasion and the expression is associated with metastasis in several malignancies <sup>100,101</sup>. High primary tumor expression of ANXA1 was associated with invasive depth, TNM-stage and both lymph node and distant metastasis in gastric adenocarcinoma <sup>100</sup>. Studying expression of ANXA1 in nearly 200 breast cancers, tumoral overexpression of ANXA1 was associated with poor prognostic factors <sup>101</sup>.

In vitro, ANXA1 overexpression was related to resistance of chemotherapy. The sensitivity to anti-cancer drugs was increased by depletion of ANXA1 in tumor cells <sup>102</sup>.

In the Human Protein Atlas (HPA)-project a wide variety of ANXA1 expression was found in healthy and tumor cells from different tissues ranging from absence of expression to upregulated levels <sup>92</sup>. As with CUBN, ANXA1 was chosen due to its highly specific expression both in normal kidney and in RCC ([www.proteinatlas.org](http://www.proteinatlas.org)) <sup>92</sup>.

## Pyruvate kinase liver and red blood cells (PKLR)

Pyruvate kinase liver and red blood cells (PKLR) is a membrane protein expressed under normal conditions in renal tubules, hepatocytes and hematopoietic cells <sup>103</sup>. It is one of the isoforms of pyruvate kinase (PK), an enzyme which plays an important role in the finale step of the glycolysis where one phosphate group is transferred from phosphoenolpyruvate to adenosine diphosphate (ADP) resulting to production of adenosine triphosphate (ATP). All the isoforms of PK are tissue-specific: PKM1 is expressed in muscles, hearts and brains and PKM2 in most normal adult tissues <sup>104</sup>.

Mutations in the PKLR gene on chromosome 1 is associated with PK deficiency in which glycolysis is slowed down and cells need to use anaerobic glycolysis to product energy. In red blood cells, which lack mitochondria,

the insufficient ATP production can lead to hemolysis and later to anemia<sup>105</sup>. In the HPA-project, PKLR was found having not only a specific expression in normal tissues mentioned above but also exhibiting an exclusive expression pattern, besides hepatocellular carcinoma, in RCC (www.proteinatlas.org)<sup>92</sup>.

There is no evidence of PKLRs function in cancer but for one of the other isoforms, a prognostic value in patients with RCC was demonstrated. Elevated levels of circulating PKM2 was significantly associated with tumor stage and grade and increased levels before nephrectomy indicated a higher risk of recurrence of RCC<sup>106</sup>.

## Epidermal growth factor, latrophilin and seven transmembrane domain-containing protein 1 (ELTD1)

Epidermal growth factor (EGF), latrophilin and seven transmembrane domain-containing protein 1 belongs to adhesion G-protein-coupled receptor (GPCR) family and consists of a large extracellular domain with EGF-like repeats, a seven-transmembrane domain and a cytoplasmic tail<sup>107</sup>. ELTD1 was first identified in cardiomyocytes and smooth muscle cells of vessels and bronchioles and later on as a biomarker in tumor vessels for high grade glioma<sup>107,108</sup>.

Upregulated angiogenesis is acquired for both primary tumor growth and metastasis. Tumor associated vessels are not only many, but they are also structurally and functionally abnormal and ineffective. They are leaky and heterogeneous and branch irregularly in a chaotic network. The inner layer of vessels consisting of endothelial cells (EC) have wide junctions. These junctions allow for influx of interstitial fluid resulting in high pressure, thereby causing poor nutrient and oxygen delivery as well as poor waste removal. This causes a hypoxic tumor microenvironment which stimulates angiogenesis even more<sup>109</sup>. Also the metastatic spread is induced by this pathologic vasculature: tumor vessels are highly permeable which enables exit of tumor cells to the vascular system. Moreover, tumoral production of MMPs, which as previously commented degrade extracellular matrix and vascular basement membrane, leads to extravasation of tumor cells to distant sites<sup>98</sup>.

As commented before, one of the major regulators of angiogenesis is VEGF. The well-studied function of upregulated VEGF in a hypoxic environment such as in tumor cells leads to imbalance between pro- and anti-angiogenetic signals. This is in contrast to angiogenesis during normal conditions such as wound healing where there is an elegant balance between inhibiting and activating signals. The anti-VEGF therapy aims to turn down the VEGF drive and normalize the tumor vasculature to produce functional

vessels. Both drug delivery and oxygen transport to the tumor and the micro-environment improves by more mature vessels <sup>110</sup>.

The expression of ELTD1 is suggested to be upregulated by VEGF-A <sup>108</sup>.

The proposed regulative roll of ELTD1 in both physiological and tumor angiogenesis is based on a study published in 2013. Analyzing a large number of different tumor types and normal tissues in a TMA, ELTD1 expression was increased in tumor-associated ECs in renal and colorectal cases. Higher levels of ELTD1 in renal cell ECs was associated with increased microvasculature density (MVD) levels generating a less hypoxic environment. This resulted in smaller tumor size and better prognosis overall <sup>111</sup>. However, silencing ELTD1, both in vivo and in vitro, inhibited tumor growth by reducing MVD and tumor tissue proliferation and by increasing hypoxia, ECs apoptosis and pericyte coverage <sup>111</sup>.

# Aims of the thesis

## Overall aim

To study proteins with a specific intratumoral expression pattern in RCC and their potential to act as predictive biomarkers for two targeting agents widely used in mRCC patients.

## Specific aims

- Paper I        To evaluate the putative value of tumoral expression of CUBN as a predictive marker for sunitinib and sorafenib treatment in mRCC patients.
- Paper II        To investigate the value of ANXA1 tumoral expression alone and in combination with CUBN as a predictive marker for sunitinib therapy in mRCC patients.
- Paper III        To study the tumoral expression of PKLR alone and in combination with CUBN as a predictor for sunitinib and sorafenib therapy in mRCC patients.
- Paper IV        To examine the predictive potential of ELTD1 expression on tumor vessels for sunitinib treatment in mRCC patients.

# Materials

## Paper I, II, III and IV

The Ethics Review Board in Uppsala approved the study (2009/139). Written informed consent was obtained from patients still alive.

The TMA cohort is based on retrospectively collected data of one hundred thirty nine (139) RCC patients diagnosed between 2006 and 2010 at seven Departments of Oncology in Sweden: Uppsala (n=48), Göteborg (n=36), Örebro (n=19), Västerås (n=12), Gävle (n=11), Falun (n=7) and Karlstad (n=6). All the patients had underwent nephrectomy and were thereafter treated with various therapeutic agents for metastatic mRCC. Progression free survival was selected as the primary endpoint and calculated as time between the date of initiation of treatment to the date of clinically and/or radiologically defined progression, treatment discontinuation due to unacceptable toxicity, date of death or date of last follow-up. Overall survival as the secondary endpoint was calculated from the date of diagnosis of mRCC.

The clinical characteristics of the whole TMA population are summarized in Table 3. The majority of the cases were of clear cell carcinoma subtype (83%) as expected. Most of the patients were male (n=100, female n= 39) and middle-aged with median 65 years at diagnosis of mRCC. Fifty-two percent were diagnosed with metastatic stage of the disease already at primary diagnosis.

Table 3. *TMA patient characteristics*

<b>Characteristics</b>	<b>Case number</b>
Total patients	139
<b>Sex (%)</b>	
Male	100 (72)
Female	39 (28)
<b>Age at diagnosis RCC, years</b>	
Median	63
Range	33-77
<b>Age at diagnosis mRCC, years</b>	
Median	65
Range	34-84
<b>Histologic subtype, n (%)</b>	
Clear cell carcinoma	115 (83)
Papillary	6 (4)
Chromophobe	2 (1)
Mixed phenotype	5 (4)
Unclassified	11 (8)
Localized disease at diagnosis, n (%)	66 (48)
Metastatic disease at diagnosis, n (%)	73 (52)
<b>Time to diagnosis of metastatic disease</b>	
Median	2
Range	0-18
Metastasis < 1 year after diagnosis, n (%)	27 (40)
Metastasis > 1 year after diagnosis, n (%)	39 (60)

## Paper I and III

The patients (n=136) treated with sunitinib or sorafenib in the first or second line were selected for the studies in Paper I and III. These patients received sunitinib or sorafenib as primary therapy except from twenty patients who were treated upfront with IFN- $\alpha$ <sup>112</sup>.

In Paper I and III study, 77 patients were treated with sunitinib and 59 were treated with sorafenib. The majority of the patients, 85 percent (n=116), were medicated with these TKIs as the frontline therapy. Totally 30

patients (22%), 16 of the sunitinib treated and 14 of the sorafenib treated, suffered from severe early side effects during the first 4 weeks of the therapy. These patients were excluded from analysis because response to the TKI treatment could not be evaluated. The 106 remaining patients (sunitinib n= 61, sorafenib n= 45) were included in the final cohort in Paper I and III.

Paper I and III cohort consisted of 77 males and 29 females. Patients were in median 62.5 years (range 33-77) at the time of RCC diagnosis and 65 years (range 34-84) at the time of mRCC diagnosis. Clear cell carcinoma was the dominant histologic type (84%, n= 89) followed by papillary type (4%). The 49 patients (46%) with localized RCC were diagnosed with metastatic cancer in median 2 years later (range 0-18). Fifty four percent (n=57) had a metastatic disease already at diagnosis.

Patients who experienced a PFS of only  $\leq 3$  months were defined as the non-responding group in Paper I.

## Paper II and IV

The 99 patients treated with sunitinib as the first (n= 70) or second line (n= 29) therapy were selected for Paper II and IV study. Patients medicated in second line were previously treated with sorafenib (n=22) or IFN- $\alpha$  (n=7). Early side effects, which lead to the termination of treatment within the first 4 weeks, as in Paper I and III, excluded 22 patients (22%) from the analyses. The remaining 77 patients were included in the final cohort in Paper II and IV.

The Paper II and IV cohort consisted of 53 males and 24 females. Patients were in median 62 years (range 40-76) at the time of RCC diagnosis and 64.5 years (range 40-77) at the time of mRCC diagnosis. Eighty eight percent (n= 68) of the patients had a clear cell carcinoma. The 36 patients (47%) with localized RCC were diagnosed with metastatic cancer in median 2 years later (range 0-18). Fifty three percent (n=41) had a metastatic disease already at diagnosis.

# Methods

## Paper I, II, III and IV

### TMA generation

The TMA was constructed in co-operation with HPA ([www.proteinatlas.org](http://www.proteinatlas.org)) and all the standards used in HPA were essentially accomplished in TMA generation, immunohistochemistry and slide scanning<sup>74,92</sup>. All steps of the procedure were performed at the Swedish Science for Life Laboratory in the Department of Immunology, Genetics and Pathology of Uppsala University.

Corresponding HE slides were first examined and representative areas from the primary renal tumors were punched and brought into recipient paraffin blocks to construct TMA containing two cores with a diameter of 1 mm per tumor.

### Immunohistochemistry Paper I, II and III

Immunohistochemical staining was performed as previously in detail described<sup>112,113</sup>. Primary antibody towards CUBN, ANXA1 and PKLR (HPA004133 and HPA011272, HPA006653, Atlas Antibodies AB, Stockholm, Sweden) were validated for immunohistochemistry (IHC) according to established criteria<sup>74</sup>.

### Immunohistochemistry and slide scanning in Paper IV

Tissue microarray sections were deparaffinized in xylene, hydrated in graded alcohols and blocked for endogenous peroxidase in 0.3% hydrogen peroxide diluted in 95% ethanol. For antigen retrieval, a Decloaking chamber® (Biocare Medical, Walnut Creek, CA) was used. Slides were immersed and boiled in Citrate buffer®, pH6 (Lab Vision, Fremont, CA) for 4 min at 125C and then allowed to cool to 90C (the whole cycle is approximately 40 minutes). Automated immunohistochemistry was performed using an Autostainer 480 instrument® (Thermo Fischer Scientific, Waltham, Massachusetts, United States). The primary rabbit polyclonal antibody towards ELTD1, CD34 and VEGFR2 (HPA025229, CAB000018, CAB004028, Atlas Antibodies, Stockholm, Sweden) were diluted in 1:1000 UltraAb Diluent (Thermo Fisher Scientific) followed by incubation for 30 min at room tem-

perature (RT). The slides were further incubated with the secondary reagent anti-rabbit/mouse horse reddish peroxidase-conjugated UltraVision (Thermo Fischer Scientific) for 30 min at RT, and developed for 10 min using Diaminobenzidine (DAB) Quanto (Thermo Fisher Scientific) as chromogen. All incubations were followed by rinse in wash buffer® (Thermo Fisher Scientific) 2 X 5 min. Slides were counterstained in Mayers hematoxylin (Histolab) and cover slipped using Pertex® (Histolab) as mounting medium. High-resolution digital images were generated with an Aperio AT2 slide scanner (Aperio, Vista, CA), using a 20x objective.

### Slide scanning and evaluation of staining in Paper I, II and III

To obtain high-resolution digital images, the IHC slides were scanned with a 20x objective using the AperioScanScope XT Slide Scanner (Aperio Technologies, Vista, CA, USA).

The digital staining images were interpreted in duplicates on a colour-calibrated screen using ImageScope (Aperio, Vista, CA, USA). Staining in the tumour cells was semi-quantitatively evaluated by two independent observers, of which one pathology specialist (MN and AD). The observers were blinded to the clinical data of the patients. Disagreements were resolved by re-evaluation of the images<sup>112,113</sup>.

The membrane staining was annotated in Paper I and III. Both staining extent (circumference) and fraction (percentage) of stained cells (0-100%) were categorically estimated using a scale of 0-2 for the extent respectively 0-4 for the percentage (fraction) of cells stained.

The combined expression score is the product of the staining extent score and fraction score, resulting to a scale from 0 to 6 for membrane.

In Paper I the combined immune score 0-1 was defined as CUBN negative tumors (CUBN -) by median (2) while CUBN positive tumors (CUBN +) had score 2-6<sup>112</sup>. In Paper III, the combined immune score 0-2 was defined as PKLR negative tumors (PKLR-) by median (3) and PKLR positive tumors (PKLR+) had score 3-6.

The cytoplasmic staining was annotated in Paper II. Staining intensity was defined from negative to strong (0-3) and proportion of stained cells using a scale of 0-4 (0-100%).

As in Paper I and III, the combined expression score was calculated resulting scale from 0 to 7. In Paper II, the combined immune score 0-1 was defined as ANXA1 negative tumors (ANXA-) by median (2) while ANXA1 positive tumors (ANXA +) had score 2-7<sup>113</sup>.

### Image analysis in Paper IV

Instead of manual assessment, a mechanized scoring was chosen for Paper IV<sup>114</sup>. Colour deconvolution with Fiji software was used in image analysis

<sup>115</sup>. The ELTD1-, CD34- and VEGFR2-positive area percentage of each TMA core was measured from the generated DAB images by a CellProfiler pipeline based on colour-thresholding <sup>116</sup>.

The ELTD1-positive area percentage as compared to the total tumor area was calculated in Paper IV and the median expression (1.1%) classified patients into ELTD1 low (expression < 1.1 %) and as ELTD1 high (expression  $\geq$  1.1%). The expression varied from 0.3 to 5.6 % and was exclusively noted in tumor vessels. The vessel expression of CD34 and VEGFR2 was also evaluated in Paper IV and according to the median expression (0.7%/0.9%) patients were categorized into CD34 high/low and VEGFR2 high/low groups.

# Results

## Paper I

The 61 sunitinib and 45 sorafenib treated patients in the Paper I cohort experienced a PFS of 7 months in median (range 0.5-40 months). Overall survival was in median 26.5 months (range 1-144 months).

Fifty percent (50%) of the tumors (n= 53) were CUBN positive. CUBN positive patients experienced a better PFS compared to CUBN negative patients (PFS median 8 months versus 4 months,  $p= 0.0019$ ).

Median OS did also differ significantly between patients with CUBN expression and patients with lack of expression. CUBN positive patients experienced a median OS of 36 months compared to CUBN negative patients with a median OS of 15 months ( $p= 0.00001$ ).

Significant differences in PFS for CUBN + and - expression remained when sunitinib (n=61) and sorafenib (n=45) medicated groups were analysed independently (p-values of 0.02 and 0.03 respectively).

In the non-responding group (n=28, 26%), i.e. patients treated with  $\leq 3$  months with sunitinib or sorafenib, the fraction of patients with no CUBN expression was significantly higher compared to patients with PFS  $> 3$  months ( $p= 0.028$ ).

## Paper II

The 77 sunitinib treated patients in the final study cohort had a PFS of 7 months in median (range 0.5-34 months). Overall survival was in median 29 months (range 1-108 months).

Thirty-two percent (32%) of tumors (n=25) were ANXA1 negative. Median PFS differed significantly between ANXA1 positive and ANXA1 negative patients. ANXA1 negative patients experienced a median PFS of 9 months compared to ANXA1 positive patients with a median PFS of 6.5 months ( $p= 0.02$ ).

Patients with lack of ANXA1 expression experienced improved OS compared to patients with expression (OS median 31 months versus 26 months,  $p= 0.0047$ ).

An extended analysis studying first and second line sorafenib treated patients (n=53) in our TMA failed to show any correlation between cytoplasmic ANXA1 expression and PFS/OS (p= 0.43/0.88).

In another subanalysis the combined expression of cytoplasmic ANXA1 and membranous CUBN from Paper I was studied (n= 77). The 23 patients with the combination of ANXA1 (+) and CUBN (-) tumor had a significantly shorter PFS compared to other combinations of ANXA1 and CUBN-expression. The combination of these two markers yielded a higher predictive value than ANXA1 alone (p = 0.0017, 2 groups analysis; p= 0.0038, 4 groups analysis). Patients with ANXA1 positive and CUBN negative tumor (n=23) were treated with sunitinib in median for three months, patients with ANXA1 and CUBN negative tumor (n= 12) for seven months, patients with ANXA1 and CUBN positive tumor (n= 29) for seven months and patients with ANXA1 negative and CUBN positive tumor (n= 13) for nine months.

### Paper III

By categorizing patients by median into two groups, 55/106 (52%) of patients had a high PKLR expression and 51/106 (48%) of patients had a low PKLR expression.

PKLR + patients had an improved PFS in comparison with PKLR – patients. The median PFS for the first group was 8 months and for the latter group 5 months (p= 0.019). A significant difference was also seen in OS analysis: patients with PKLR expression experienced an OS of 31 months versus 17 months (median) in patients with lack of PKLR expression (p=0.001).

In a subanalysis studying the combined expressions of our previously investigated potential predictor CUBN together with PKLR we found a higher predictive value. Patients with expression of neither CUBN nor PKLR in their primary tumor were treated with sunitinib and sorafenib for 4 months in median. The median PFS was doubled (8 months) in patients with tumor membranous expression of CUBN or PKLR or both (p=0.007).

### Paper IV

By categorizing patients by median in to two groups, 38/77 (50%) of patients had low and 39/77 (50%) of patients had a high intratumoral ELTD1 vessel expression.

High ELTD1 expression was significantly associated with longer PFS. The median PFS for these patients was 8 months versus PFS of 5.5 months for patients with low ELTD1 expression (p = 0,017). The same significant correlation was noticed in OS, patients with high ELTD1 expression had a

median OS of 31 months compared to patients with low ELTD1 expression with OS in median 24 months ( $p = 0.03$ ).

To study if the number of vessels has any predictive value for sunitinib-treatment CD34 and VEGFR2 positive area fraction was also calculated and showed no correlation with either PFS or OS ( $p = 0.15/0.77$  and  $0.62/0.85$ ).

In a subanalysis the expression of ELTD1 was studied in first and second line sorafenib treated group ( $n = 53$ ) and no association with PFS or OS was found in these patients ( $p = 0.67$  versus  $0.79$ ).

# Discussion

The prognosis for mRCC patients is still poor despite the advances in the treatment during the last decade. Approximately 25 to 30 % of the patients with localized disease are diagnosed with recurrence during the first two years after nephrectomy <sup>3</sup>. About 20% of all RCC patients are diagnosed with metastatic disease up-front <sup>1</sup>. Hence, there is still a compelling need to improve the oncological treatment. The standard oncological treatments, chemotherapy and radiotherapy, have not been successful for mRCC patients <sup>3</sup> and the previously used cytokine therapy with IL-2 and IFN- $\alpha$  results in few objective responses and have many, some even severe, side-effects <sup>35,36</sup>.

After the involved pathways activated in RCC were clarified, one major goal of research through the past decade has been to test different molecular targets as therapy options. VEGF- and PDGF-receptors are overexpressed in clear cell RCC, the most common type of RCC, as a result of tumor-suppressor gene VHL inactivation. Due to VHL mutation, high levels of HIF are accumulated and stimulate production of proangiogenic factors, such as VEGF <sup>117</sup>. Oral TKIs, for example sunitinib and sorafenib, target in particular VEGF- and PDGF-receptors on endothelial cells and changed the standard treatment of mRCC. Tyrosine kinase inhibitors, especially sunitinib, are still, in the era of upcoming immunotherapy, considered as the up-front therapy for mRCC and are also used in subsequent therapy lines. One of the first large studies showed that sunitinib given as first line treatment in metastatic disease significantly extended PFS to 11 months compared to the other group which was treated with IFN- $\alpha$  <sup>37</sup>. In another study comparing sorafenib with placebo in second line, PFS was significantly longer (5.5 months) in the sorafenib treated group <sup>37,39</sup>. At the same time, TKIs have a range of unpleasant, adverse and severe side effects and the costs of the treatment remains a challenge. Furthermore, it is important to acknowledge that some patients are more or less refractory to these treatments <sup>117,118</sup>. Therefore, predictive markers are needed to guide treatment choice in routine clinical practise. Which patients will benefit from these therapeutic agents?

The established markers for patients with mRCC can only help to estimate prognosis <sup>18</sup>. This is a well-studied field and several prognostic markers have been defined. There is however a greater need to find markers predicting treatment benefit, not the least for the targeting agents widely used in mRCC

patients today. The most efforts to find such predictors has focused on proteins in serum meanwhile published TMA-based studies are in general minor than ours as earlier described. In the largest tumor tissue based study, a higher tumor cell PD-L1 expression was observed as a negative predictor for sunitinib and pazopanib response<sup>80</sup>.

Since no such biomarkers for TKIs are in clinical use the main aim of this thesis was to assess potential candidates and hopefully find significant associations with treatment benefit. With this purpose we built a TMA where tumor cores from 139 RCC cases were sampled. Proteins with a specific and differential expression pattern in RCC were chosen in a unique co-operation with HPA-project with their excellent knowledge of human proteins. Additionally, in view of TKIs vessel targeting mode of action, we also chose one protein, ELTD1, with known expression on endothelial cells. These selected proteins were evaluated for prediction of treatment benefit to sunitinib and sorafenib. As we defined PFS as the primary endpoint the retrospective design had no negative impact on our studies.

In Paper I and III we investigated two proteins, CUBN and PKLR, which both have a tissue-specific expression in the renal tubules in the healthy kidney as well as in RCC<sup>92</sup>. The role of these proteins in cancer is unknown, though both CUBN and one isoform of PK, PKM2, seem to have a prognostic value in RCC<sup>93,106</sup>. A high tumoral membrane expression in 106 sunitinib or sorafenib treated mRCC patients was significantly associated with longer PFS. In Paper I, CUBN positive patients were observed with a doubled PFS (median eight months) compared to CUBN negative patients (median four months). The same pattern was repeated in Paper III where patients with PKLR membranous expression of their primary RCC tumor were treated in median for eight months with sunitinib or sorafenib compared to five months for patients with lack of PKLR expression.

Selecting the minor group of patients with no effect of TKIs could minimize the number of patients suffering from side effects, allocate these patients to other treatment options and thereby also have an economical benefit. Therefore, we studied non-responders further in Paper I, i.e. patients treated  $\leq 3$  months with sunitinib or sorafenib (n=28), and noticed a significantly higher fraction of CUBN negative tumors in this group of patients.

After these observations, we combined the expression of CUBN and PKLR to investigate whether a higher predictive value could be reached. As we expected, the patients with the combination of CUBN and PKLR negative primary tumors were treated significantly shorter with sunitinib or sorafenib (median PFS of four months) in contrast to patients having both or one of these proteins expressed (median PFS 8 months). Therefore we conclude that the combined expression analysis of these two proteins seem to improve the patient selection. According to our study, the lack of both tu-

moral CUBN and PKLR expression might identify the minor group of non-responders.

In paper II we moved from the cell membrane into the cytoplasm and investigated the role of tumoral ANXA1 as a potential predictor for treatment with TKIs. Annexin A1 acts as a regulator of inflammation, balancing towards adequate activation of immune response<sup>96</sup>. It also owns some putative tumor growth and invasive properties discussed earlier in this thesis<sup>97,99</sup>. Prognostic unfavourable factors have been observed to correlate with ANXA1 expression<sup>101</sup>. In Paper II, based on 77 first or second line sunitinib treated patients, the lack of cytoplasmic ANXA1 expression in the primary tumor was significantly associated with longer PFS. We speculate that the plausible explanation could be that sunitinib cannot overcome ANXA1s angiogenic drive. In patients with less ANXA1 expression, sunitinib might accomplish its function and this patient group obviously benefits more from the treatment. As in Paper I and III, sorafenib treated patients were also analysed for a possible predictive role in Paper II but no significant associations with survival was found for ANXA1. We conclude that ANXA1 is a specific marker for sunitinib. The function of TKIs differs in some extent and might therefore require their own predictors.

We further studied whether the combination of the markers explored in Paper I and II, CUBN and ANXA1, could also add predictive value for sunitinib therapy compared to each marker alone. In line with our previous results, the combined ANXA1 positive and CUBN negative expression showed significantly shorter PFS compared to other combinations of expression. This combined analysis may more precisely select the patients with no gain from the sunitinib medication.

While angiogenesis is one of the primary steps for tumor growth and metastasis we decided to study a specific vessel associated protein, ELTD1, in Paper IV. Epidermal growth factor, latrophilin and seven transmembrane containing protein-1 is known to be upregulated in tumor ECs and is considered as an important regulator of angiogenesis. Using the same patient cohort as in Paper II (77 patients with sunitinib treatment) we found that high ELTD1 expression on tumor vessels is a positive predictor for sunitinib therapy. Patients with high ELTD1 tumoral expression experienced significantly longer PFS. The same correlation could not be recalled for sorafenib treated patients and indicates that ELTD1 expression do not generally predict response to all TKIs. Moreover, we studied expression of CD34, a known vasculature pan-endothelial marker widely expressed in both normal and tumor vessels, and VEGFR2, the most prominent VEGFR for VEGF-induced angiogenesis and the target for sunitinib, in the same cohort. They were both exclusively expressed in tumor vessels but showed no predictive potential

for TKI-treatment. Therefore we conclude that the phenotype of vessels but not vessel density has predictive value.

In addition to significant associations of expression pattern of these four proteins with PFS we also found significant correlations to OS in all our studies. Though, many of the patients were medicated with other therapies, after sunitinib and sorafenib, likely affecting OS. A possible prognostic role of these proteins may also have an effect on OS. A tumor marker may only have a prognostic value, only a predictive value or both. The advantage of choosing PFS over OS as the primary endpoint is that PFS is not affected by subsequent therapies. Thereby, PFS should be a more reliable measure of the potential predictive value.

In summary, we present four promising predictors; CUBN, ANXA1, PKLR and ELTD1, for widely used TKIs in mRCC patients. Further studies are needed to confirm their predictive value but hopefully, in the future, they will give guidance to clinicians to choose the optimal therapy for each patient.

# Conclusions

Treatment with TKIs has changed the prognosis of patients with mRCC from poor to at least better. Still, making treatment choice easier, avoiding severe toxicity and reducing the costs of these expensive therapies, predictive markers are really needed. Such biomarkers could help to optimize therapy at the individual level. In this thesis we were searching for such markers through studying three different structures, membrane, cytoplasm and vessels in primary renal cell tumor tissue. Expression of CUBN, PKLR and ANXA1 in renal tumor cells as well as expression of ELTD1 on tumor associated ECs were all significantly correlated with response to TKI treatment. Our results further indicate that different combined expressions could more accurately define especially patients with no treatment benefit of sunitinib or sorafenib. With such analyses, these patients can be selected at baseline and be offered other therapy options.

## Future perspectives

Collecting data prospectively with all known prognostic factors would give information whether CUBN, ANXA1, PKLR and ELTD1 also have independent prognostic values. All data in our studies was collected retrospectively and the data of known prognostic markers in serum were missing for the majority of patients. However, finding new prognostic factors was not the aim of this thesis. We focused on responses to TKI treatment and studied PFS as the primary endpoint.

Tumoral heterogeneity is one question to have in mind. It would be interesting to study not only the primary tumor but also the current metastases because tumor cell characteristics may be different in different lesions and also change over time. TKI treatment is used in a metastatic situation and therefore it would be more accurate to test potential predictors based on such tumor material. However, in practice this is a problem. Patients are not usually biopsied at recurrence which unable predictor analysis.

The size of the cohorts always remains a challenge even though the number of patients in our studies exceeds most other published studies in the field. Also, the absence of validation in an independent cohort is a limitation. Therefore, validating our results in a larger TMA cohort would give more reliable results.

The treatment arsenal for mRCC patients is growing fast, besides sunitinib and sorafenib other therapies are routinely used and new options are already around the corner. This makes studies like ours, to collect tumor tissue which takes time, somewhat difficult to complete during this era of rapid therapy development. Investigating the expression of our four proteins in tumor tissue from mRCC patients treated with newer TKIs, such as pazopanib and cabozantinib, as well as with checkpoint inhibitors, is of high interest.

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